Supplemental Table: Methods to probe GR-DNA Interactions

Method	Type	Utility/Application	Process	Pitfalls	Reference
ChIP-seq (Chromatin immunoprecipitation followed by deep sequencing)	*in vivo*	Identify GR occupied regions (GORs) within the genome *in vivo.* Particularly useful for determining changes in occupancy in different cellular conditions (*e.g.* different cell types)	Cellular chromatin is crosslinked and fragmented by mechanical or chemical cleavage. Next an antibody bound resin is used to precipitate the target factor together with crosslinked genomic fragments, the crosslinks are reversed, and the co-precipitated DNA fragments are prepared into a library and sequenced.	Relies on the specificity of the antibody-antigen recognition as well as the context-specific exposure of the epitope, efficiency of crosslinking and reverse crosslinking, accessibility of chromatin for pulldown, and proper library preparation and normalization to cellular chromatin input. Care should be taken when interpreting ChIP-seq results, especially when making quantitative interpretations comparing ChIP-seq peak signals done in different conditions	1–6
ChIP-exo (Chromatin immunoprecipitation followed by exonuclease digestion and deep sequencing)	*in vivo*	Identify GR occupied regions (GORs) within the genome *in* vivo with base pair resolution on a genome-wide scale	Similar general protocol as ChIP-seq except it uses an endonuclease to degrade accessible DNA before the crosslinking is reversed. Protein-bound DNA is protected from cleavage and, upon sequencing, reveals genomic occupied sites at base pair resolution.	The pitfalls associated with ChIP-seq apply here. Additionally, differences in exonuclease properties can lead to nuclease-specific artifacts and altered ChIP-exo "footprint."	4,7,8
DNase-seq (DNaseI hypersensitive site (DHS) sequencing)	*in vivo*	Identify chromatin regions that are most accessible to nuclease cleavage by DNaseI throughout the genome. These regions, referred to as "nucleosome-depleted," are thought to be "open chromatin" often important in regulation and occupied by TRFs and other non-nucleosomal proteins	Low concentrations of DNaseI are added to permeabilized cells or isolated nuclei. Open, or "nucleosome-depleted" regions of chromatin are more sensitive to cleavage by the enzyme. These can be detected genome-wide by deep sequencing.	Relies on permeabilization of cells or isolation of nuclei - both of which are inefficient steps that can create bias. Although less biased than other nucleases, DNaseI may have some sequence specificity that could influence DNA cleavage.	6,9–13
DNaseI footprinting	*in vitro*	Determine *in vitro* protein–DNA binding affinity and sequence specificity	High affinity protein–DNA interactions typically protect DNA from cleavage by DNaseI, resulting in a protein-specific "footprint" of protected DNA with an intensity of protection roughly proportional to the fractional occupancy of the protein at the binding site. Labeled PCR amplified DNA is incubated with variable amounts of purified protein. The complexes are digested with limiting amounts of DNaseI and the footprint is visualized via PAGE.	Normally performed *in vitro* on unchromatinized DNA. For GR, predominantly limited to DBD, however, recently done on full-length purified GR in a limited number of conditions.	14–16

FAIR-seq (Formaldehyde-Assisted Isolation of Regulatory Elements and deep sequencing)	*in vivo*	Alternate method to identify "nucleosome-depleted" regions of chromatin	This procedure identifies "open chromatin" based on the observation that nucleosome rich regions of the genome are more efficiently crosslinked by formaldehyde than nucleosome depleted regions. Briefly, genomic DNA is crosslinked, the DNA is then fragmented and phenol chloroform extracted to segregate nucleosome-bound (organic phase) from unbound (aqueous phase). "Nucleosome-depleted" DNA is identified via deep sequencing.	Limited use with GR in different cell types and under different physiological conditions with some notable exceptions.	17,18
X-ray Crystallography	*in vitro*	Structural analysis of GR–DNA Interactions	Obtain three-dimensional structure from exposing a crystal of protein–DNA complex to an x-ray beam. The diffraction pattern intensities obtained can be used to determine structure factors and calculate electron density maps from which structures can be derived.	For GR, work has been done with isolated domains and not with full-length receptor. X-ray crystallography is not optimal for intrinsically disordered proteins and almost half of GR is disordered. This causes issues with obtaining large quantities of purified full-length receptor required for crystallography. Additionally, crystallization can stabilize non-physiological conformations; hence, derived structures, especially protein–protein interaction surfaces, should be considered as models requiring validation through other tests.	19–25
NMR (Nuclear magnetic resonance)	*in vitro*	Probing of the specific chemical environment experienced by specific atoms within a protein–DNA complex.	NMR makes use of the particular magnetic properties of atomic nuclei to allow for the study of dynamic features of the protein-DNA interaction. Different labeling strategies can be used, either the protein or DNA is labeled with a heavy isotope, such as ^{15}N or ^{13}C. Spectra of individual residues or DNA bases can be used to compare protein alone to DNA bound, and vice-versa.	For GR, NMR analysis has been conducted with isolated DBD bound to different GR binding sequences. High concentrations of protein necessary for analysis precludes study of full-length GR.	26–30
Molecular dynamics	*in silico*	Monitor the computer-simulated movement of atoms within a macromolecule in different states and ask how a structural model behaves under different perturbations.	Computer-modeled movements of atoms and molecules within a macromolecule are constrained by computed inter-particle forces and potential energies, interatomic potentials, and molecular mechanics force fields. Simulations occur over short, fixed time intervals and give information about dynamics within a macromolecule.	Molecular dynamics has been used to monitor how the GR-DBD interacts with different DNA sequences. This has been conducted with isolated domains as these are the only structural models available.	30–34

HDX-MS (Hydrogen deuterium exchange mass spectrometry)	*in vitro*	Used to identify changes in surface exposed regions of GR under different signaling contexts. These changes in solvent accessibility can be used to infer changes in conformation upon change of signaling context (*e.g.* ligand or DNA binding).	Deuterium exchanges more rapidly with solvent exposed amide hydrogens and slower with regions buried within the core of the protein or covered upon interaction with a partner. In this method, protein is incubated in a deuterated environment to allow amide hydrogens to exchange; then the reaction is quenched, the protein is digested with the acid protease pepsin, and is subjected to liquid chromatography mass spectrometry (LC-MS) to determine the amount of deuterium uptake, and thus solvent accessibility, of each proteolytic cleavage product.	HDX with GR-DNA interactions has been conducted with isolated domains. Magnitude of changes in solvent accessibility do not scale directly with changes in conformation or dynamics, for example small changes in solvent accessibility can be due to large changes in conformation. As GR forms a homodimer, it is difficult to determine the degree to which each sister subunit is undergoing a change is solvent accessibility.	35–37
FP (Fluorescence Polarization)	*in vitro*	GR–DNA Binding Assay	Monitor binding of proteins to fluorescently labeled oligonucleotide probe. A fluorophore, generally attached to the smaller of the two reactants (a short DNA fragment in this case), is excited with polarized light. Upon binding to protein, the combined mass of the complex increases, slowing the tumbling of the fluorophore. This decrease in tumbling rate is measured as a change in the intensity of fluorescence emission at a particular angle relative to the initial polarized excitation.	For GR–DNA interactions, FP has been conducted with full length GR and with isolated domains using purified proteins or extracts. Fluorescent label may affect the labeled DNA conformation or influence protein–DNA binding.	38,39
EMSA (Electrophoretic Mobility Shift Assay)	*in vitro*	GR–DNA Binding Assay	Monitor binding of proteins to labeled an oligonucleotide probe. A protein–DNA complex will migrate more slowly on a non-denaturing polyacrylamide gel. Antibodies can also be used to target the protein of interest to generate a supershifted protein-antibody-DNA band.	*In vitro* assay that is useful for determining apparent equilibrium binding affinity, but can be difficult to quantify the kinetics of the protein–DNA complex. Has been conducted with full-length GR and with isolated domains using purified proteins or extracts.	21,27,28,40
Luciferase Reporter Assays	*in vivo*	Monitor transcriptional activity change in response to GR–genome interactions	Generally, a plasmid bearing a GOR-containing fragment cloned upstream of a minimal promoter and the luciferase reporter gene is transfected into cells where endogenous or overexpressed GR can bind to the GOR fragment and potentially stimulate or repress transcription of the luciferase reporter gene. The amount of luciferase reporter made can be measured by adding the substrate luciferin to cells. The luciferase enzyme will then catalyze a reaction that	Uses highly abundant exogenous DNA that does not reflect native chromatin states, endogenous nucleosomal packing, histone modifications, *etc*. Vulnerable to numerous artifacts upon TRF protein overexpression and gene dosage. Typically tests a single GOR fragment in conjunction with a non-native promoter. Does not account for post-RNA polymerase II initiation events, which have been shown to be highly regulated in endogenous contexts.	21,28,37,40,41

			produces oxyluciferin and light, which is used to infer transcriptional activity.		
Precise genome editing (CRISPR-Cas, TALEN, and ZFN)	*in vivo*	Deletion, or single base pair resolution manipulation of potential GREs at endogenous loci. Used in conjunction with ChIP, qPCR, RNA-seq, transcriptomics, or other *in vivo* assays, can validate GRE activity and identify GRE target gene.	Each of these technologies uses a DNA sequence specific nuclease directed to a particular genomic site (e.g. a potential GRE). After cleavage of the endogenous locus, endogenous cellular machinery repairs the DNA break. Non-homologous end joining can introduce small insertions and deletions mutating the endogenous sequence. Alternatively, when a repair template is provided, the cells can be steered towards using homology driven repair to introduce predetermined sequences with single base pair precision.	Although powerful methods, TALEN and ZFN technologies have proven to be time consuming and expensive routes to obtaining desired precisely edited genomic elements. CRISPR-Cas systems have shown great promise to precisely edit potential GREs as well as introduce affinity or fluorescent protein tags, and inducible degradation signaling sequences to endogenous TRF gene bodies. All of these technologies have low but finite rates of off-target editing.	2,42
Fluorescence Microscopy	*in vivo*	Monitor GR-DNA Interactions in cells	In general, fluorescently tagged GR is expressed in a cell line of choice and its localization is monitored. This basic technique has been combined with Number and Brightness methodologies to infer the oligomeric status of GR on chromatin. This technique has also been combined with fluorescence recovery after photobleaching (FRAP) to analyze GR-chromatin dynamics.	Adding a large fluorescent tag to a protein may inhibit natural function, or have stress inducing / toxic effects on cells. Tagged proteins are overexpressed and strongly altered stoichiometries can produce various artifacts. Inference of oligomerization states seems particularly vulnerable to such artifacts.	43–48

References

1. Landt, S. G. *et al.* ChIP-seq guidelines and practices of the ENCODE and modENCODE consortia. *Genome Res.* **22,** 1813–1831 (2012).
2. Telorac, J. *et al.* Identification and characterization of DNA sequences that prevent glucocorticoid receptor binding to nearby response elements. *Nucleic Acids Res.* **44,** 6142–6156 (2016).
3. Uhlenhaut, N. H. *et al.* Insights into Negative Regulation by the Glucocorticoid Receptor from Genome-wide Profiling of Inflammatory Cistromes. *Mol. Cell* **49,** 158–171 (2013).
4. Starick, S. R. *et al.* ChIP-exo signal associated with DNA-binding motifs provides insight into the genomic binding of the glucocorticoid receptor and cooperating transcription factors. *Genome Res.* **25,** 825–835 (2015).
5. Reddy, T. E. *et al.* Genomic determination of the glucocorticoid response reveals unexpected mechanisms of gene regulation. *Genome Res.* **19,**

2163–2171 (2009).

6. John, S. *et al.* Chromatin accessibility pre-determines glucocorticoid receptor binding patterns. *Nat. Genet.* **43,** 264–268 (2011).
7. Rhee, H. S. & Pugh, B. F. Comprehensive genome-wide protein-DNA interactions detected at single-nucleotide resolution. *Cell* **147,** 1408–1419 (2011).
8. Lim, H. *et al.* Genomic redistribution of GR monomers and dimers mediates transcriptional response to exogenous glucocorticoid in vivo. *Genome Res.* **25,** 836–844 (2015).
9. Gross, D. S. & Garrard, W. T. Nuclease hypersensitive sites in chromatin. *Annu. Rev. Biochem.* **57,** 159–97 (1988).
10. Crawford, G. E. Genome-wide mapping of DNase hypersensitive sites using massively parallel signature sequencing (MPSS). *Genome Res.* **16,** 123–131 (2005).
11. Burd, C. J. & Archer, T. K. Chromatin architecture defines the glucocorticoid response. *Mol. Cell. Endocrinol.* **380,** 25–31 (2013).
12. John, S. *et al.* Interaction of the glucocorticoid receptor with the chromatin landscape. *Mol. Cell* **29,** 611–624 (2008).
13. Koohy, H., Down, T. A. & Hubbard, T. J. Chromatin accessibility data sets show bias due to sequence specificity of the DNase I enzyme. *PLoS One* **8,** e69853 (2013).
14. Bain, D. L. *et al.* Glucocorticoid receptor-DNA interactions: Binding energetics are the primary determinant of sequence-specific transcriptional activity. *J. Mol. Biol.* **422,** 18–32 (2012).
15. Robblee, J. P., Miura, M. T. & Bain, D. L. Glucocorticoid Receptor–Promoter Interactions: Energetic Dissection Suggests a Framework for the Specificity of Steroid Receptor-Mediated Gene Regulation. *Biochemistry* **51,** 4463–4472 (2012).
16. De Angelis, R. W., Maluf, N. K., Yang, Q., Lambert, J. R. & Bain, D. L. Glucocorticoid Receptor-DNA Dissociation Kinetics Measured in Vitro Reveal Exchange on the Second Time Scale. *Biochemistry* **54,** 5306–5314 (2015).
17. Giresi, P. G., Kim, J., McDaniell, R. M., Iyer, V. R. & Lieb, J. D. FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) isolates active regulatory elements from human chromatin. *Genome Res.* **17,** 877–885 (2007).
18. Burd, C. J. *et al.* Analysis of chromatin dynamics during glucocorticoid receptor activation. *Mol. Cell. Biol.* **32,** 1805–1817 (2012).
19. Shi, Y. A glimpse of structural biology through X-ray crystallography. *Cell* **159,** 995–1014 (2014).
20. Luisi, B. F. *et al.* Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* **352,** 497–505 (1991).
21. Meijsing, S. H. *et al.* DNA Binding Site Sequence Directs Glucocorticoid Receptor Structure and Activity. *Science* **324,** 407–410 (2009).
22. Hudson, W. H., Youn, C. & Ortlund, E. A. The structural basis of direct glucocorticoid-mediated transrepression. *Nat. Struct. Mol. Biol.* **20,** 53–58

(2013).

23. Bledsoe, R. K. *et al.* Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. *Cell* **110,** 93–105 (2002).
24. He, Y. *et al.* Structures and mechanism for the design of highly potent glucocorticoids. *Cell Res.* **24,** 713–26 (2014).
25. Schoch, G. A. *et al.* Molecular Switch in the Glucocorticoid Receptor: Active and Passive Antagonist Conformations. *J. Mol. Biol.* **395,** 568–577 (2010).
26. Campagne, S., Gervais, V. & Milon, A. Nuclear magnetic resonance analysis of protein-DNA interactions. *J. R. Soc. Interface* **8,** 1065–1078 (2011).
27. Watson, L. C. *et al.* The glucocorticoid receptor dimer interface allosterically transmits sequence-specific DNA signals. *Nat. Struct. Mol. Biol.* **20,** 876–883 (2013).
28. Thomas-Chollier, M. *et al.* A naturally occuring insertion of a single amino acid rewires transcriptional regulation by glucocorticoid receptor isoforms. *Proc. Natl. Acad. Sci.* **110,** 17826–17831 (2013).
29. Schöne, S. *et al.* Sequences flanking the core-binding site modulate glucocorticoid receptor structure and activity. *Nat. Commun.* **7,** 12621 (2016).
30. Hudson, W. H. *et al.* Distal substitutions drive divergent DNA specificity among paralogous transcription factors through subdivision of conformational space. *Proc. Natl. Acad. Sci. U. S. A.* **113,** 326–331 (2016).
31. Karplus, M. & McCammon, J. A. Molecular dynamics simulations of biomolecules. *Nat. Struct. Biol.* **9,** 646–52 (2002).
32. Bredenberg, J. & Nilsson, L. Conformational states of the glucocorticoid receptor DNA-binding domain from molecular dynamics simulations. *Proteins Struct. Funct. Genet.* **49,** 24–36 (2002).
33. Eriksson, M. A., Härd, T. & Nilsson, L. Molecular dynamics simulations of the glucocorticoid receptor DNA-binding domain in complex with DNA and free in solution. *Biophys. J.* **68,** 402–426 (1995).
34. Stockner, T., Sterk, H., Kaptein, R. & Bonvin, A. M. J. J. Molecular dynamics studies of a molecular switch in the glucocorticoid receptor. *J. Mol. Biol.* **328,** 325–334 (2003).
35. Konermann, L., Pan, J. & Liu, Y.-H. Hydrogen exchange mass spectrometry for studying protein structure and dynamics. *Chem. Soc. Rev.* **40,** 1224–1234 (2011).
36. Kirschke, E., Goswami, D., Southworth, D., Griffin, P. R. & Agard, D. A. Glucocorticoid Receptor Function Regulated by Coordinated Action of the Hsp90 and Hsp70 Chaperone Cycles. *Cell* **157,** 1685–1697 (2014).

37. Khan, S. H. *et al.* Binding of the N-terminal Region of Coactivator TIF2 to the Intrinsically Disordered AF1 Domain of the Glucocorticoid Receptor Is Accompanied by Conformational Reorganizations. *J. Biol. Chem.* **287,** 44546–44560 (2012).
38. Zhang, H., Wu, Q. & Berezin, M. Y. Fluorescence anisotropy (polarization): from drug screening to precision medicine. *Expert Opin. Drug Discov.* **10,** 1145–1161 (2015).
39. Shah, N. & Scanlan, T. S. Design and evaluation of novel nonsteroidal dissociating glucocorticoid receptor ligands. *Bioorg. Med. Chem. Lett.* **14,** 5199–5203 (2004).
40. Surjit, M. *et al.* Widespread Negative Response Elements Mediate Direct Repression by Agonist- Liganded Glucocorticoid Receptor. *Cell* **145,** 224–241 (2011).
41. Hua, G., Paulen, L. & Chambon, P. GR SUMOylation and formation of an SUMO-SMRT/NCoR1-HDAC3 repressing complex is mandatory for GC-induced IR nGRE-mediated transrepression. *Proc. Natl. Acad. Sci.* **113,** E626–E634 (2016).
42. Ran, F. A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8,** 2281–2308 (2013).
43. Unruh, J. R. & Gratton, E. Analysis of molecular concentration and brightness from fluorescence fluctuation data with an electron multiplied CCD camera. *Biophys. J.* **95,** 5385–5398 (2008).
44. McNally, J. G. The Glucocorticoid Receptor: Rapid Exchange with Regulatory Sites in Living Cells. *Science* **287,** 1262–1265 (2000).
45. Presman, D. M. *et al.* DNA binding triggers tetramerization of the glucocorticoid receptor in live cells. *Proc. Natl. Acad. Sci.* **113,** 8236–8241 (2016).
46. Presman, D. M. *et al.* Live Cell Imaging Unveils Multiple Domain Requirements for In Vivo Dimerization of the Glucocorticoid Receptor. *PLoS Biol.* **12,** e1001813 (2014).
47. Stavreva, D. A., Muller, W. G., Hager, G. L., Smith, C. L. & McNally, J. G. Rapid Glucocorticoid Receptor Exchange at a Promoter Is Coupled to Transcription and Regulated by Chaperones and Proteasomes. *Mol. Cell. Biol.* **24,** 2682–2697 (2004).
48. Kino, T., Liou, S.-H., Charmandari, E. & Chrousos, G. P. Glucocorticoid receptor mutants demonstrate increased motility inside the nucleus of living cells: time of fluorescence recovery after photobleaching (FRAP) is an integrated measure of receptor function. *Mol. Med.* **10,** 80–88 (2006).